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SOLID PHASE METHOD FOR SYNTHESIS PEPTIDE-SPACER-LIPID CONJUGATES,
CONJUGATES SYNTHESIZED THEREBY AND TARGETED LIPOSOMES
CONTAINING THE SAME

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FIELD OF THE INVENTION

[0001] The present invention is related to solid phase synthesis methods for preparing peptide-spacer-lipid conjugates and uses of the conjugates.

BACKGROUND OF THE INVENTION

[0002] Drug delivery plays a crucial role in the improvement of agents for therapeutic treatment, since many agents have unfavorable drawbacks if they are directly applied to a human body. Therefore, developing a delivery system is necessary for a particular agent to improve its availability such as reduction of side effects, enhancement of efficacy, and convenience in usage. For example, antineoplastic chemotherapies are limited by adverse side effects resulting from their widespread toxicity to normal tissues. Therefore, a delivery system which could prevent drug diffusion and concentrate the drug to the disease site is required.

[0003] Liposomes can provide several advantages for use as a drug delivery system for the reasons that they are safe to a biological system, have an excellent spherical bilayer for carrying either hydrophilic or hydrophobic drugs, and can prevent drugs from degradation and diffusion. Moreover, liposomes can be modified to have additional functions for specific purposes. A successful example is shown as polyethylene-glycol-grafted (PEG-grafted) liposomes. These modified liposomes can evade the reticuloendothelial system and have prolonged circulation time in blood. Furthermore, cytotoxic cancer drugs encapsulated in the PEG-grafted liposomes provide a remarkable enhancement in anti-tumor activity effect and decrease the side effect of the toxicity to the normal cells. The PEG-grafted liposomes thereby gained commercial application and opened the possibility for further modification of these PEG-grafted liposomes for targeted delivery.

[0004] Several types of targeted liposomes have been developed (Maruyama *et al.*, *Biochim Biophys Acta*. 1995, 1234, 74-80; and Allen TM, *Trends Pharmacol Sci*. 1994, 15, 215-220). Commonly used targeted liposomes include (1) targeting ligands linked at the lipid headgroups on the conventional liposomes (Type A); (2) targeting ligands linked at the lipid headgroups on the PEG-grafted liposomes (Type B); and (3) targeting ligands attached at the distal end of the PEG chain on the PEG-grafted liposomes (Type C). To date, studies have shown that targeted liposomes of Type C provide a better liposomal structure for targeted delivery (Maruyama *et al.*, *Biochim Biophys Acta*. 1995, 1234, 74-80). Based on this liposomal structure, several types of molecules, such as antibodies (Ahmad *et al.*, *Cancer Res*. 1993, 53, 1484-8; and Suzuki *et al.*, *Biochim Biophys Acta*., 1995, 1245, 9-16), proteins (Eavarone *et al.*, *J Biomed Mater Res*. 2000, 51, 10-4) small synthesis molecules (Gabizon *et*

al., *Bioconjug Chem.* 1999, 10, 289-98) and peptides (Zalipsky *et al.*, *Bioconjug Chem.* 1997, 8, 111-8), have been developed as the targeting ligands for binding the target sites. Among these types of molecules, peptides are considered as highly potential targeting ligands, since a peptide can serve as a recognition component in protein-protein interactions such as receptor-ligand interactions. Furthermore, many cellular membrane receptors associated with diseases have been studied.

[0005] Peptides, such as RGD-peptides, somatostatin, chemotactic peptides, vasoactive intestinal peptide, and mimetics thereof, are good candidates as the targeting ligands. Many counter receptors of these peptides have been found being overexpressed in various tumor cells. Moreover, peptides and peptide mimetics have several unique advantages over other types of molecules (e.g., antibodies). Generally, these peptides bind to target cells with a ligand-receptor association at high affinity and enter the intercellular compartments through receptor-mediated endocytosis. However, an antibody-based targeted liposome may not utilize the endocytosis pathway into the interior of the cells by the antigen on the cell membrane. Furthermore, peptides have less opportunity to be recognized by the reticuloendothelial system and are, thus, cleared from the blood circulation system. Peptide mimetics can provide a higher binding affinity and a better resistance to the proteases degradation than natural peptides.

[0006] Currently, two approaches for preparing peptide-based targeted liposomes have been developed, whereby the peptide ligands can be attached at the distal end of PEGs. The first approach is incorporating end-group functionalized PEG-lipid conjugates into liposomes and then conjugating with peptide ligands (Zalipsky *et al.*, *Bioconjug. Chem.*, 1995, 6, 705-8).

However, when the end-group functionalized PEGs are conjugated to peptide ligands, a non-homogeneous conjugation may happen if there is more than one reaction group in the peptide ligands. Furthermore, the unreacted end-groups of functionalized PEG are difficult to define and are completely deactivated after the coupling reaction. The second approach is directly incorporating the peptide-PEG-lipid conjugates into liposomal membranes (Zalipsky *et al.*, *Bioconjug. Chem.*, 1997, 8, 111-8). This approach can provide a structurally well-defined targeted liposome component.

[0007] Although peptide-PEG-lipid conjugates are the expected molecules for preparing the targeted liposomes, the available conjugates are still very limited and the synthesis is difficult. This is so, because, in the peptide-PEG-lipid conjugates, the chemical property of the side chains in peptides is diverse, the molecular mass of PEG is heterogeneous, and the nature of lipids is amphiphilic. These properties cause difficulty in the synthetic processes of side chain protection, purification, and reaction and is evident in that very few peptide-PEG-lipid conjugates have been synthesized and in that the conjugation of a peptide, a spacer, and a lipid often induce the formation of a clumsy linker and an unusual functional group.

[0008] Zalipsky *et al.* (1997) discloses the method for synthesizing Tyr-Ile-Gly-Ser-Arg-PEG-lipid conjugates (SEQ ID NO 001: Tyr-Ile-Gly-Ser-Arg). However, this method cannot be used as a general method for synthesizing a broad range of peptide-PEG-lipid conjugates as a nonspecific reaction of bromoacetyl group with strong nucleophilic residues, such as an amino group or other thiol group, in the peptide may occur. Furthermore, in the Tyr-Ile-Gly-Ser-Arg-PEG-lipid conjugate, a thioacetyl (-S-CH₂-CO-) linker was used for conjugating the peptide and PEG, which is unfavorable in industrialization since an additional modification at

the ends of peptide and PEG to a bromoacetyl group and a thiol group, respectively, is required. The urethane linkage between PEG and lipid in the Tyr-Ile-Gly-Ser-Arg-PEG-lipid conjugate is unnatural and acid-base labile. Therefore, a need exists for a synthesis method of preparing a broad range of peptide-spacer-lipid conjugates.

BRIEF SUMMARY OF THE INVENTION

[0009] The present invention provides a convenient solid phase synthesis method for preparing peptide-PEG-lipid conjugates and provides various linkage groups (such as amide group) for conjugating a peptide, a spacer and a lipid. According to the solid phase synthesis method of the present invention, several advantages can be achieved, such as a simplified synthesis, an automated synthesis, a facile purification process in each reaction step, and minimized product losses during synthesis. In addition, the present synthesis method is suitable for preparing a wide range of peptide-spacer-lipid conjugates.

[0010] The present invention also provides a peptide-spacer-lipid conjugate, prepared by the present solid phase synthesis method. The peptide-spacer-lipid can be incorporated into a liposome as the targeting moiety for liposomal drug delivery to specific cells.

[0011] The present invention also provides a targeting liposome comprising the present peptide-spacer-lipid conjugate.

ABBREVIATION LIST

[0012] The present invention is herein disclosed using the following chemical nomenclature:

2-Br-Cbz	2-bromobenzyloxycarbonyl
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2-Br-Z	2-bromobenzyloxycarbonyl
2-Cl-Cbz	2-chlorobenzyloxycarboyl
2-Cl-Z	2-chlorobenzyloxycarbonyl
Abu	4-aminobutyric acid
AC	acetyl
Acm	acetamidomethyl
Boc	t-butyloxycarbonyl
Bz	benzoyl
Bzl	benzyl
Cbz	benzyloxycarboyl
DCC	dicyclohexylcarbodiimide
DC-Chol	3 β [N-(N',N'-dimethylaminoethane)carbamyl] cholesterol
DCM	Dichloromethane
DDAB	dimethylammonium bromide
Dde	1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl
DIPCDI	1,3-diisopropylcarbodiimide

DMAP	dimethylaminopyridine
DME	ethylene glycol dimethyl ether
DMF	N, N-dimethylformamide
DMRIE bromide	N-[1-(2,3-ditetradecyloxy)propyl]-N,N-dimethyl-N hydroxyethylammonium
DMS	dimethylsulfide
DOPAT	1,2-dioleoyloxy-3-(trimethylamino) propane
DOPE	dioleoyl phosphatidylethanolamine
DORIE bromide	N-[1-(2,3-dioleoyloxy)propyl]-N,N-dimethyl-N-hydroxy ethylammonium
DOTMA	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
DOX	doxorubicin
DSPE	distearyl phosphatidylethanolamine
EDC	1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide
EDT	ethanedithiol
EGF	epidermal growth factor
FGF	fibroblast growth factor

Fmoc	9-fluorenylmethyloxycarbonyl
For	formyl
HF	hydrogen fluoride
HGF	hepatocyte growth factor
HOBt	N-hydroxybenzotriazole
HSPC	hydrogenated soybean phosphatidylcholine
IGF	insulin-like growth factor
Im	imidazol-1-yl
MBHA	4-methylbenzhydramide
MeOH	methanol
Mmt	4-methoxytrityl
Mtr	4-methoxy-2,3,6-trimethylbenzene-sulfonyl
Mts	mesitylene-2-sulfonyl
Mtt	4-methlytrityl
mPEG-DSPE	methoxypolyethylene glycol-distearyl phosphatidylethanolamine
NGF	nerve growth factor

NHS	N-hydroxysuccinimide
PACAP	pituitary adenylate cyclase-activating peptide
Pbf	2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl
PDGF	platelet-derived growth factor
Pd-C	Palladium supported on active carbon catalysts
PEG	polyethylene glycol
pMeoBzl	p-methoxybenzyl
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl
pNP	para-nitrophenyl
SPPS	solid phase peptide synthesis
SST	somatostatin
Su	succinimide
TCP	2,4,5-trichlorophenyl
TEA	triethylamine
TFA	trifluoroacetic acid
TFE	trifluoroethanol

TFMSA	trifluoromethanesulfonic acid
Tf	trifluoromethanesulfonyl
Tfa	trifluoroacetyl
TGF	transforming growth factor
THP	tetrahydropyranyl
Tos	tosyl
Trt	trityl
tBu	tert-butyl
tButhio	tert-butylthio
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide
Z	benzyloxycarbonyl.

DETAILED DESCRIPTION OF THE INVENTION

I. Solid Phase Synthesis Method for Preparing Peptide-Spacer-Lipid Conjugates

[0013] According to the present invention, the solid phase synthesis method for preparing a peptide-spacer-lipid conjugate comprises the steps of:

- (1) synthesizing an amino acid residue protected peptidyl resin in solid phase;

- (2) conjugating a spacer and a lipid to the peptidyl resin, thereby forming a peptide-spacer-lipid resin having a peptide-spacer-lipid;
- (3) cleaving the peptide-spacer-lipid from the peptide-spacer-lipid resin;
- (4) removing at least one side chain protecting group from at least one amino acid of the peptide-spacer-lipid, thereby forming a peptide-spacer-lipid conjugate; and
- (5) subjecting the peptide-spacer-lipid conjugate to a process selected from a group consisting essentially of:

no further processing,

modifying a peptide portion of the peptide-spacer-lipid conjugate to a cyclic form during any of the foregoing steps (1) – (4), and

modifying a peptide portion of the peptide-spacer-lipid conjugate to a cyclic form after any of the foregoing steps (1) – (4).

A. Solid Phase Synthesis of Amino Acid Residue Protected Peptidyl Resin

[0014] The peptidyl resin of the present invention may be prepared by any solid phase synthetic techniques known in the art. The relevant techniques such as Merrifield, *J. Am. Chem. Soc.*, 85, 2149 (1963), Stewart, *Solid Phase Peptide Synthesis* (Freeman and Co., San Francisco, (1969)), Stewart *et al.*, *Solid Phase Peptide Synthesis* (Pierce Chemical Company, Rockford, (1984)), and Atherton *et al.*, *Solid Phase Peptide Synthesis: A Practical Approach* (IRL Press, Oxford (1989)) are incorporated herein by reference in their entirety.

[0015] According to the present invention, Fmoc and Boc solid phase peptide synthesis (SPPS) methods are the preferred methods for preparing the peptidyl resin. The Boc SPPS uses an acid-labile Boc (1-butyloxycarbonyl) group as the protecting alpha-amino group, whereas a Fmoc SPPS uses a base-labile Fmoc (9-fluorenylmethyloxycarbonyl) group as the protecting alpha-amino group. The Fmoc and the Boc SPPS are known in the art, for example, Stewart *et al.*, Solid Phase Peptide Synthesis, Pierce Chemical Company, Rockford (1984), and Chan and White, Fmoc Solid Phase Peptide Synthesis: a Practical Approach, Oxford University Press, Oxford (2000).

[0016] After the initial attachment, the excess reagent and the by-product are washed by a washing solution. Subsequent amino acids are added to elongate the peptide chain by the process comprising the steps of: (1) deprotecting the alpha-amino protecting group with a deprotecting reagent; and (2) coupling of the amino acid with a coupling reagent in an organic solvent. A washing step with a washing solution is performed after each deprotecting and coupling step. A Kaiser test (Kaiser *et al.*, *Anal Biochem.* 1970, 34, 595-8) can be used to determine whether the coupling reaction has been completed. The coupling reaction is terminated when the test is “negative.” After the desired peptide is completed, the resulting peptidyl resin is then conjugated with a spacer.

[0017] According to the present invention, the coupling agent can be selected from the reagents for peptide bond formation. Examples of such coupling reagents include, but not limited to, dicyclohexylcarbodiimide/N-hydroxybenzotriazole (DCC/HOBt), 1,3-diisopropylcarbodiimide/N-hydroxybenzotriazole (DIPCDI/HOBt), and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide/N-hydroxysuccinimide (EDC/NHS). A

preferred embodiment of the coupling agent is DIPCDI/HOBt.

[0018] According to the present invention, the deprotecting agents for a t-butyloxycarbonyl (Boc) protecting group can be trifluoroacetic acid (TFA) and the deprotecting agent for a 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group can be piperidine.

[0020] According to the present invention, the primary solvents used for deprotecting, coupling, and washing include, but are not limited to, dichloromethane (DCM) and N,N-dimethylformamide (DMF).

Resins

[0021] According to the present invention, various resins can be used for synthesis of peptidyl resin. The resins suitable for Fmoc solid phase peptide synthesis (SPPS) include, but are not limited to, hydroxymethyl resin, Wang resin, 2-Chlorotrityl chloride resin, and Rink amide resin. The resins suitable for Boc SPPS include, but are not limited to, Merrifield resin, 4-methylbenzhydrylamide (MBHA) resin, and oxime resin. The Wang resin and the hydroxymethyl resin can be used for synthesizing the peptides having a carboxylic acid ($-\text{COOH}$), alkylamides ($-\text{C}(\text{O})\text{NHR}$), di-(alkyl)amides ($-\text{C}(\text{O})\text{NR}^1\text{R}^2$), or esters ($-\text{C}(\text{O})\text{OR}$) at a C-terminus in the Fmoc chemistry. The Rink amide resin can be used for synthesizing the peptides having an amide ($-\text{C}(\text{O})\text{NH}_2$) at a C-terminus in the Fmoc chemistry. A 2-Chlorotrityl chloride resin can be used for synthesizing a peptide having carboxylic acid, amine, or hydroxyl functional group at a C-terminus in the Fmoc chemistry. Merrifield resins can be used for synthesizing the peptides having a carboxylic acid or esters at a C-terminus in the Boc chemistry. MBHA resins can be used for synthesizing the peptides having an amide

at a C-terminus in the Boc chemistry. An oxime resin can be used for synthesizing the peptides having alkylamides or esters at a C-terminus in the Boc chemistry.

[0022] In a preferred embodiment of the present invention, the synthesis of a peptide-spacer-lipid conjugate is performed by using a Wang resin, a 2-Chlorotrityl chloride resin, and a Rink amide resin in the Fmoc chemistry.

Amino protecting groups

[0023] According to the present invention, the amino group in an amino acid, which is used in the peptide chain elongation, can be protected during the amino acid coupling to the elongating peptide. After the coupling reaction, the protecting group is removed for the next amino group-protected amino acid coupling.

[0024] According to the present invention, the suitable protecting groups include, but are not limited to, acyl type protecting groups such as formyl, trifluoroacetyl, and acetyl; aromatic urethane type protecting groups such as Fmoc, benzyloxycarbonyl (Cbz), and substituted Cbz; aliphatic urethane protecting groups such as t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, and cyclohexyloxycarbonyl; and alkyl type protecting groups such as benzyl and triphenylmethyl. However, the Fmoc and the Boc are the preferred protecting groups.

Side chain protecting group

[0025] According to the present invention, a side chain protecting group is directed to a group which can be attached to the side chain of an amino acid for protecting the side chain during chemical reactions, but which can also be easily removed after the required reactions. The

suitable side chain protecting groups for the amino group include, but are not limited to, acetyl (AC), Boc, Cbz, 2-chlorobenzyloxycarbonyl (2-Cl-Cbz), 2-bromobenzyloxycarbonyl (2-BrCbz), 4-methytrityl (Mtt), benzyloxycarbonyl (Z), Fmoc, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde), and trifluoroacetyl (Tfa). The suitable side chain protecting groups for a hydroxyl group include, but are not limited to, benzyl (Bzl), tert-butyl (tBu), and trityl (Trt). The suitable side chain protecting groups for a thiol group include, but are not limited to, acetamidomethyl (Acm), Bzl, tBu, tert-butylthio (tButhio), p-methoxybenzyl (pMeoBzl), and 4-methoxytrityl (Mmt). The suitable side chain protecting groups for a phenolic hydroxyl group include, but are not limited to, tetrahydropyranyl, tBu, Trt, Bzl, Cbz, z-Br-Cbz, and 2,5-dichlorobenzyl. The suitable side chain protecting groups for an imidazole include, but are not limited to, Boc, Mtt, tosyl (Tos), and Trt. The suitable side chain protecting group for an indole can be, but is not limited to, Boc. The suitable side chain protecting groups for a carboxylic acid include, but are not limited to, benzyl, 2,6-dichlorobenzyl, tBu, and cyclohexyl. The suitable side chain protecting groups for a guanidino group include, but are not limited to, 4-methoxy-2,3,6-trimethylbenzene-sulfonyl (Mtr), mesitylene-2-sulfonyl (Mts), 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl (Pbf), 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc), and Tos.

B. The Synthesis of Lipid-Spacer-Peptidyl Resin

[0026] In a preferred embodiment of the present invention, the lipid-spacer-peptidyl resin is synthesized by conjugating a spacer to the peptidyl resin to obtain a spacer-peptidyl resin and then conjugating a lipid to the spacer-peptidyl resin.

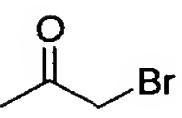
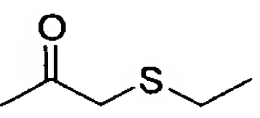
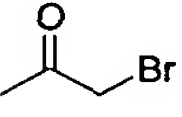
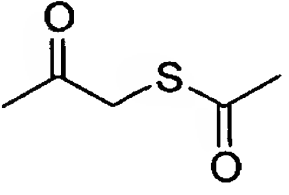
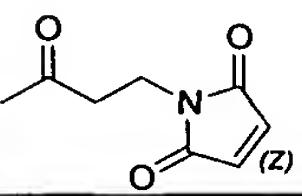
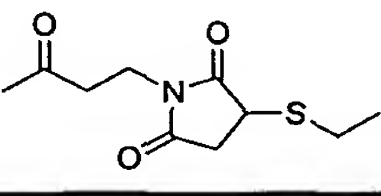
[0027] In another embodiment of the present invention, the lipid-spacer-peptidyl resin is

synthesized by conjugating a spacer-lipid to the peptidyl resin.

Conjugation of the Spacer to the Peptidyl Resin

[0028] According to the present invention, a hydrophilic polymer spacer can be conjugated to the peptidyl resin through various linkage functional groups. Examples of the linkage functional groups are listed in Table I as follows.

Table I. Functional groups of N-terminals of peptides, heads of lipids, and ends of spacers for producing the linkage functional groups

N-terminal groups of peptides or headgroups of lipids	End groups of spacers	Linkage functional groups
—NH_2	$\text{activated HO—}\overset{\text{O}}{\parallel}\text{C—}$ activating agents: DCC/DMAP, DCC/HOBT DIPCDI/HOSu	$\text{—NH—}\overset{\text{O}}{\parallel}\text{C—}$
—NH_2	$\text{X—CH}_2\text{—}$ X=halide, Tos, Tf	$\text{—NH—CH}_2\text{—}$
—NH_2	$\text{R—O—}\overset{\text{O}}{\parallel}\text{C—O—}$ $\text{R=Im, pNP, Su, TCP}$	$\text{—NH—}\overset{\text{O}}{\parallel}\text{C—O—}$
	$\text{HS—CH}_2\text{CH}_3$	
	HS—C(=O)—CH_3	
	$\text{HS—CH}_2\text{CH}_3$	

[0029] Typically, a linking reaction is performed by coupling an end-group functionalized or activated spacer to the peptidyl resin in a suitable solvent, and shaking at a temperature in a range of approximately 0 °C to approximately 90 °C until the Kaiser test is “negative.” After the reaction has been completed, the excess reagents and the by-products are removed by a washing solution, and then the peptidyl resin is subjected to coupling with the lipid.

[0030] In a preferred embodiment of the present invention, the end-group functionalized spacer is a functionalized polyethylene glycol (PEG). A review for the preparation of various end-group functionalized or activated PEG is shown in the art of Zalipsky S., *Bioconjug. Chem.*, 6, 150-165 (1995).

[0031] Conjugation of PEG to the peptidyl-resin requires a suitable functional group at the end of the PEG and the N-terminal of the peptide. When an amine (-peptide-NH-PEG) is the linkage functional group, a PEG with an end-group functionalized by a halide (e.g., -Cl, -Br, and -I) or a sulfonate (e.g., -OSO₂C₆H₄CH₃, -OSO₂CH₂CF₃) can be used to couple with the amino group at the N-terminal of the peptidyl resin. When a urethane (-peptide-NHC(O)O-PEG) is the linkage functional group, a PEG with an end-group functionalized by an active carbonate (e.g., -C(O)-Im, -OC(O)-pNP, -OC(O)-NHS, -OC(O)-TCP) can be used to couple with the amino group at the N-terminal of the peptidyl resin. When an amide (-peptide-NHC(O)-PEG) is the linkage functional group, a PEG with the end-group functionalized by the activated carboxyl group (e.g., the carboxyl group activated by DCC/HOBt, DCC/dimethylaminopyridine (DMAP), DIPCDI/HOBt, and EDC/NHS) can be used to couple with the amino group at the N-terminal of the peptidyl resin. When a thio ester (-peptide-C(O)CH₂SC(O)-PEG) is the linkage functional group, a PEG with the end-group

functionalized by the thio acid (-PEG-C(O)S) can be used to couple with the N-terminal of the peptidyl resin in which the N-terminal of the peptidyl resin is modified to bromoacetyl (resin-peptide-C(O)CH₂Br). When a thio ether (-peptide-C(O)CH₂SCH₂-PEG) is the linkage functional group, a PEG with the end-group functionalized by the thiol group (-PEG-CH₂SH) can be used to couple with the N-terminal of the peptidyl resin in which the N-terminal of the peptidyl resin is modified to bromoacetyl (resin-peptide-C(O)CH₂Br). When the thio ether of a maleimido/thio conjugate is the linkage functional group, a PEG with the end-group functionalized by a thiol group (C(O)-PEG-C(O)CH₂CH₂SH) can be used to couple with the N-terminal of the peptidyl resin in which the N-terminal of the peptidyl resin is modified to the maleimido group (maleimido-CH₂CH₂C(O)-peptide-resin).

[0032] The suitable solvents for the coupling reaction can be selected from a group consisting essentially of DCM, chloroform, DMF, tetrahydrofuran (THF), and different ratios of mixtures thereof.

[0033] The washing solutions can be selected from a group consisting essentially of DCM, chloroform, methanol (MeOH), DMF, THF, hydrogen cyanide, water, buffers, and different ratios of mixtures thereof.

[0034] In a preferred embodiment of the present invention, the linkage functional group is an amide bond that is a carboxylic group functionalized PEG conjugating to the N-terminal amino group of the peptidyl resin. In the amide bond coupling reaction, the activating agent for carboxyl group in the reaction is selected from a group consisting essentially of the reagents used in peptide bond formation, such as DCC/HOBt, DIPCDI/HOBt or EDC/NHS. The suitable solvents for the coupling reaction are selected from a group consisting

essentially of DCM, chloroform, DMF, THF, and different ratios of mixtures thereof. The washing solutions are selected from a group consisting essentially of DCM, chloroform, MeOH, DMF, THF, hydrogen cyanide, water, buffers, and different ratio of mixtures thereof. The reaction temperature is in a range of approximately 20°C to approximately 90°C. The preferred activating agent for carboxylic group is DIPCDI/HOBt and the solvent is selected from a group consisting essentially of DCM, chloroform, and DMF. The preferred washing solutions are selected from a group consisting essentially of chloroform, MeOH, water, DMF, buffers (pH 3.0-11.0), and different ratios of mixtures thereof. The preferred reaction temperature is in a range of approximately 20°C to approximately 60°C.

Conjugation of Lipid to the Spacer-Peptidyl Resin

[0035] According to the present invention, a lipid is conjugated to a spacer-peptidyl resin through linkage functional groups as above described. Typically, the coupling reaction is performed by adding a lipid to an end-group functionalized or activated spacer-peptidyl-resin in a suitable solvent and shaking in a temperature range of approximately 0°C to approximately 90°C for a duration of up to approximately 24 hours. After the reaction is completed, the excess reagents and the by-products are removed by washing solutions.

[0036] In an embodiment of the present invention, the spacer in a spacer-peptidyl-resin is PEG. Functionalization of the end-group of PEG is above recited with respect to Zalipsky S., *Bioconjug. Chem.*, 6, 150-165 (1995).

[0037] Conjugation of a lipid to a spacer-peptidyl-resin requires a suitable functional group at the end of PEG and the headgroup of the lipid. When an amine (lipid-NH-PEG-) is the

linkage functional group, a PEG with an end-group functionalized by a halide (e.g., -Cl, -Br, and -I) or sulfonate (e.g., -OSO₂C₆H₄CH₃, -OSO₂CH₂CF₃) can be used to couple with the amino group in the headgroup of the lipid. When a urethane (lipid-NHC(O)O-PEG-) is the linkage functional group, a PEG with end-group functionalized by active carbonate (e.g., -C(O)-Im, -OC(O)-pNP, -OC(O)-Su, -OC(O)-TCP) can be used to couple with the amino group in the headgroup of the lipid. When an amide (lipid-NHC(O)-PEG-) is the linkage functional group, a PEG with the end-group functionalized by an activated carboxyl group (e.g., the carboxyl group activated by DCC/HOBt, DCC/DMAP, DIPCDI/HOBt, EDC/NHS) can be used to couple with the amino group in the headgroup of the lipid. When a thio ester (lipid-C(O)CH₂SC(O)-PEG-) is the linkage functional group, a PEG with the end-group functionalized by a thio acid (-spacer-C(O)S) can be used to couple with the lipid in which the headgroup is modified to a bromoacetyl (lipid-C(O)CH₂Br). When thio ether (lipid-C(O)CH₂SCH₂-PEG-) is the linkage functional group, a PEG with the end-group functionalized by a thiol group (-spacer-CH₂SH) can be used to couple with the lipid in which the headgroup is modified to a bromoacetyl is modified to bromoacetyl (lipid-C(O)CH₂Br). When the thio ether of a maleimido/thio conjugate is the linkage functional group, a PEG with the end-group functionalized by a thiol group (-PEG- CH₂SH) can be used to couple with the lipid in which the headgroup is attached to a maleimido group (maleimido-CH₂CH₂C(O)-lipid).

[0038] In a preferred embodiment of the present invention, the conjugation of a lipid with PEG-peptidyl resin is formed by an amide bond, whereby a carboxylic group at the terminus of the PEG-peptidyl resin is linked to the amino group in the headgroup of the lipid. In

general, the coupling reaction initiates by adding an activating agent to activate the end carboxyl group of the carboxyl-PEG-peptidyl resin. A lipid is then added to the activated carboxyl-PEG-peptidyl resin in a suitable solvent with a base, and then the mixture is shaken under nitrogen in a temperature range of approximately 0°C to approximately 90°C. After the reaction has been completed, the excess reagents and the by-products are removed by washing solutions.

[0039] The activating agent for a carboxyl group in the reaction can be selected from a group consisting essentially of the reagents used in peptide bond formation, such as DCC/HOBt, DIPCDI/HOBt, or EDC/HOSu. The suitable solvents for the coupling reaction can be selected from a group consisting essentially of DCM, chloroform, DMF, THF, and different ratios of mixtures thereof. The washing solutions can be selected from a group consisting essentially of DCM, chloroform, MeOH, DMF, THF, hydrogen cyanide, water, buffers, and different ratio of mixtures thereof. The reaction temperature is in a temperature range of approximately 20°C to approximately 90°C. The preferred activating agent for a carboxylic group in the reaction is EDC/NHS. A preferred base in the coupling reaction is triethylamine (TEA). The preferred solvent in the coupling reaction is a mixture of chloroform and DMF. The preferred reaction temperature is in a range of approximately 45°C to approximately 85°C.

Conjugation of the Spacer-Lipid to the Peptidyl Resin

[0040] According to the present invention, the lipid-spacer-peptidyl resin can be synthesized by a process comprising the steps of: (1) preparing an end-group functionalized spacer-lipid conjugate; and (2) coupling the conjugate with the peptidyl resin. The end-group

functionalized spacer-lipid conjugate can be synthesized by conjugating an end-group functionalized spacer to a lipid. In one embodiment of the present invention, the end-group functionalized spacer is a functionalized PEG. A review for the preparation of various end-group functionalized or activated PEG can be seen in the art of Zalipsky S., *Bioconjug. Chem.* 6, 150-165 (1995). Examples for synthesizing an end-group functionalized PEG-lipid conjugate is described in Blume *et al.*, *Biochim. Biophys. Acta*, 1149, 180-184 (1993) and Zalipsky, *Bioconjugate Chem.*, 4, 269-299 (1993).

[0041] According to the present invention, the end-group functionalized PEG-lipid conjugate can be coupled with peptidyl resin following the above described methods.

C. Cleavage of Peptide-Spacer-Lipid from Resin

[0042] According to the present invention, cleavage of peptide-spacer-lipid from resin is achieved by shaking a cleavage reagent with a lipid-spacer-peptidyl resin. In general, the cleavage reagents and procedure used in the present invention are the same as the treatment used in the art of SPPS.

[0043] When a Wang resin, a 2-Chlorotrityl chloride resin, and a Merrifield resin are used to synthesize the peptide-spacer-lipid conjugates with a carboxyl group at a C-terminus, the lipid-spacer-peptidyl resin can be cleaved by a cleavage reagent (a mixture of at least one acid, scavenger, and solvent).

[0044] The acid can be selected from a group consisting essentially of TFA, hydrogen fluoride (HF), and trifluoromethanesulfonic acid (TFMSA). The scavenger can be selected from a group consisting essentially of thioanisole, anisole, ethanedithiol (EDT),

dimethylsulfide (DMS), ethylmethanethiol, trifluoroethanol (TFE), 4-methylmercaptophenol, benzyl mercaptan, triethylsilane, and water. The suitable solvents for the cleavage of peptide-spacer-lipid include, but are not limited to, DCM, chloroform, DMF, THF, and different ratios of mixtures thereof. Cleavage of the lipid-spacer-peptide from resin requires a strong acid, such as HF or TFMSA, in the cases of Boc chemistry and TFA in Fmoc chemistry. DCM and DMF are the primary solvents used for cleavage.

[0045] When a hydroxymethyl resin or a Wang resin is used to synthesize a lower alkylamide C-terminus of the peptide-spacer-lipid conjugates, cleavage of the peptide-spacer-lipid conjugates from the resin can preferably proceed under a mixture of alkylamine, aluminum chloride, and DCM. The cleavage procedure is known in the art, such as C. R. McArthur, *et al.*, (1982), Can. J. Chem., 60, 1836, which is incorporated herein⁷⁷⁸ by reference. When hydroxymethyl resins or Wang resins are used to synthesize a lower alkylated carboxyl C-terminus of peptide-spacer-lipid conjugates, cleavage of the peptide-spacer-lipid conjugates from the resin can preferably proceed under a mixture of alkylalcohol, TEA, potassium cyanide, and benzene. The cleavage procedure is known in the art, such as Moon, *et al.*, (1994), Tetrahedron Lett., 35, 8915, which is incorporated herein by reference. When a Rink amide resin is used to synthesize an amidated carboxyl C-terminus of peptide-spacer-lipid conjugates, cleavage of the peptide-spacer-lipid conjugates from the resin can preferably proceed under a mixture of TFA, scavengers and DCM. When a MBHA resin is used to synthesize an amidated carboxyl C-terminus of peptide-spacer-lipid conjugates, cleavage of the peptide-spacer-lipid conjugates from the resin can preferably proceed under a mixture of HF and scavengers. When an oxime resin is used to synthesize an alkylamide C-terminus of

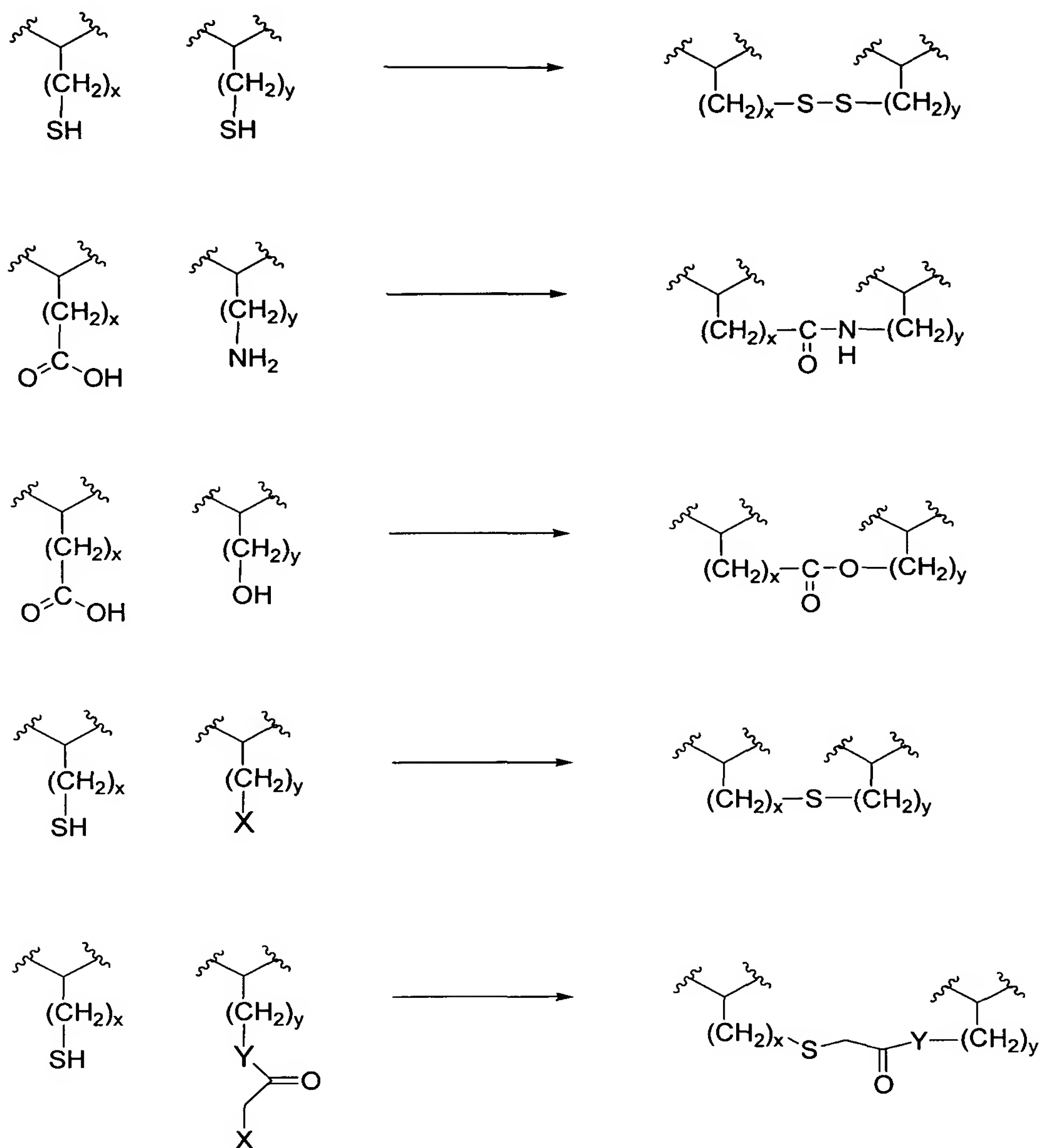
peptide-spacer-lipid conjugates, the cleavage reagent preferably used is RNH_2 . When an oxime resin is used to synthesize an alkyl ester C-terminus of peptide-spacer-lipid conjugates, the cleavage reagent preferably used is alkylalcohol and TFE.

D. Removal of Side Chain Protecting Groups

[0046] In general, the side chain protecting groups in the present invention are removed by the same process used in the art of SPPS. Most side chain protecting groups, such as t-Bu, Boc, Mts, Mmt, Pbf, Pmc, Tos, Trt, of amino acids can be removed by TFA or HF during the cleavage of the peptide-spacer-lipid from the resin. Other side chain protecting groups can be selectively removed by suitable deprotecting agents. The preferred deprotecting agents for removing Acn include, but are not limited to, Hg(II) , Ag(I) , Tl(III) , and I_2 . The preferred deprotecting agents for removing Bzl, Z, 2-bromobenzyloxycarbonyl (2-Br-Z), 2-chlorobenzyloxycarbonyl (2-Cl-Z) is palladium (Pd) supported on an active carbon catalyst (Pd-C) / hydrogen gas. The preferred deprotecting agent for removing tButhio includes thio and tributylphosphine. The preferred deprotecting agent for removing Fmoc is piperidine.

E. Modification of the Peptide Portion of the Conjugate

[0047] According to the present invention, the peptide portion of a peptide-spacer-lipid conjugate may be modified to a cyclic form by forming an intramolecular linkage between two amino acids or their derivatives in the peptide according to the method known in the art. Examples of the intramolecular linkage include, but not limited to, disulfide, amide, ester, thioether, thioacetate, and thioacetamine, which are shown as follows:



Wherein x and y represent an integer in a range of 1 to 3; X represents either Cl or Br; and Y represents either NH or O.

[0048] The intramolecular disulfide bond can be formed by using an oxidizing agent, such as I_2 , $Tl(III)$, and air, to specifically oxidize the thiol groups in the peptide. The amide and ester bond can be prepared by using a carboxyl group activator, such as DCC/HOBt, to form an

amide bond with an amino group, or to form an ester bond with a hydroxy group in the peptide. The thioether bond and alpha-substituted acetic acid linkage can be prepared by displacing the chloro or bromo group with a sulfur group. See examples of Englebrechtsen, D.R. *et al.*, *Tetrahedron Lett.*, 1995, 36, 8871-8874; Barker *et al.*, *J. Med. Chem.*, 1992, 35, 2040-2048; and Or *et al.*, *J. Org. Chem.*, 1991, 56, 3146-3149, each of which is incorporated herein by reference.

II. Peptide-Spacer-Lipid Conjugates

[0049] According to the present invention, the peptide-spacer-lipid conjugate is synthesized by the present method and is composed of a linear hydrophilic polymer chain having a linkage functional group at each end, which covalently conjugates with a peptide ligand at one end and a lipid at the other end. The peptide-spacer-lipid conjugates can be incorporated into liposomes in which the lipids of the conjugates are inserted into the bilayer of the liposomes to anchor the conjugates in the cell membranes, whereby the peptide ligands of the conjugates facilitate exposure outside of the cell membranes and facilitate selective binding to cells or tissues.

A. Peptide Ligands

[0050] According to the present invention, the peptide ligand is a synthetic peptide composed of natural amino acids. In a preferred embodiment of the present invention, the peptide ligand can bind to a receptor. The receptor can be selected from a group consisting essentially of somatostatin receptors, vasoactive intestinal peptide receptors, integrin receptors, fibroblast growth factor receptors, hepatocyte growth factor receptors, epidermal

growth factor receptors, insulin-like growth factor receptors, nerve growth factor receptors, vascular endothelial growth factor receptors, platelet-derived growth factor receptors, and transforming growth factor receptors.

[0051] In another embodiment of the present invention, the peptide ligand can be selected from a group consisting essentially of hormones, cytokines, toxins, chemotaxins, and peptides of extracellular matrix for cell adhesion.

[0052] Examples of the peptide ligands and ligand-receptor pairs are listed in Table II as follows.

Table II. Ligand-receptor pairs and examples of the peptide ligands

SEQ ID NO	Ligands	Receptor	Examples of Peptide Ligands
SST	SSTR2, 5	Octreotide:	(d)Phe-Cys-Phe-(d)Trp-Lys-Thr-Cys-Thr(ol)
		BIM-23268:	cyclic Cys-Phe-Phe-(d)Trp-Lys-Thr-Phe-Cys(NH ₂)
		BIM-23023:	cyclic (d)Phe-Cys-Tyr-(d)Trp-Lys-Abu-Cys-Thr(NH ₂)
SEQ ID NO 002	VIP	VIP/PACAP	VIP (1-12):His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg
SEQ ID NO 003			EP 0 620 008:Ala-Val-Thr-Thr-Asp-Asn-Tyr-Thr
SEQ ID NO 004		Prepro-VIP	(111-122):Ser-Ser-Glu-Gly-Glu-Ser-Pro-Gln-Phe-Pro-Glu-Glu-Leu-Glu-Lys
SEQ ID NO 005	RGD	integrins	Fibronectin CS-1:Glu-Ile-Leu-Asp-Val
SEQ ID NO 006			Fibronectin CS-3:Gly-Arg-Gly-Glu-Ser
SEQ ID NO 007			Laminin (442-447):Leu-Gly-Thr-Ile-Pro-Gly
SEQ ID NO 008	HGF	MET	HGF: Gly-His-Lys

SEQ ID NO 009	EGF	EGFR	EGF(20-31):Cys-Met-His-Ile-Glu-Ser-Leu-Asp-Ser-Tyr-Thr-Cys
SEQ ID NO 010		EGFR	USP5,969,099: Cys-Arg-Phe-Leu-Val-Gln-Asp-Lys-Xaa-Ala-Cys
SEQ ID NO 011	FGF	FGFR	FGF(1-11):Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys-Lys-Pro
SEQ ID NO 012		FGFR	FGF(119-126):Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu
SEQ ID NO 013			WO00/03245:Cys-Ser-Ala-Leu-Phe-Val-Gly-Ala-Pro-Phe-His-Val-Pro-Asp-Cys
SEQ ID NO 014		USP	5,789,382:Arg-Lys-Leu-Ala-Val-Tyr-Trp-Ser-Ser-Tyr-Lys-Arg-Ser-Arg-Tyr
SEQ ID NO 015	IGF	IGFR	IGFI(30-41):Gly-Tyr-Gly-Ser-Ser-Ser-Arg-Arg-Ala-Pro-Gln-Thr
SEQ ID NO 016		JP	601009599:Tyr-Phe-Asp-Lys-Pro-Thr-Gly-Tyr-Gly-Ser-Ser-Ser-Arg-Arg-Ala-Pro-Gln-Thr
SEQ ID NO 017	NGF	NGFR	Prepro-NGF(99-115): Pro-Glu-Ala-His-Trp-Thr-Lys-Leu-Gln-His-Ser-Leu-Asp-Thr-Ala-Leu-Arg
SEQ ID NO 018			WO97/15593:Cys-Gly-Ser-Glu-Val-Pro-Asn-Ser-Ala-Arg-Cys-Cys-Val-Cys
SEQ ID NO 019	VEGF	VEGFR:	Cys-Ser-Cys-Lys-Asn-Thr-Asp-Ser-Arg-Cys-Lys-Ala-Gly-Leu-Gly-Leu-Asn-Gly-Arg-Thr
SEQ ID NO 020	PDGF	PDGFR:	Gly-Arg-Pro-Arg-Glu-Ser-Gly-Lys-Lys-Arg-Lys-Arg-Lys-Arg-Leu-Lys-Pro-Thr

[0053] In another embodiment of the present invention, the peptide ligands are hormones. According to the present invention, the hormones include, but are not limited to, a somatostatin, vasoactive intestinal peptide (VIP), an epidermal growth factor (EGF), a fibroblast growth factor (FGF), a platelet-derived growth factor (PDGF), a nerve growth factor (NGF), a hepatocyte growth factor (HGF), a transforming growth factor (TGF), an

insulin-like growth factor (IGF), and a vascular endothelial growth factor (VEGF).

[0054] In another preferred embodiment of the present invention, the peptide ligands are peptide fragments of extracellular matrix, which bind to the integrin or laminin receptors. Examples of these peptides include, but are not limited to, the peptides containing the amino acid sequence selected from a group consisting essentially of SEQ ID NO 021: Arg-Gly-Asp, SEQ ID NO 022: Arg-Gly-Glu, SEQ ID NO 023: Asp-Gly-Glu-Ala, SEQ ID NO 024: Glu-Ile-Leu-Asp-Val, SEQ ID NO 025: Gly-Pro-Arg-Pro, SEQ ID NO 026: Lys-Gln-Ala-Gly-Asp-Val, and SEQ ID NO 027: Gln-Lys-Arg-Leu-Asp-Gly-Ser.

[0055] In another preferred embodiment of the present invention, the peptide ligands are:

EGF(20-31)

SEQ ID NO 009, Cys-Met-His-Ile-Glu-Ser-Leu-Asp-Ser-Tyr-Thr-Cys;

FGF I, Acidic Brain derived (1-11)

SEQ ID NO 011, Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys-Lys-Pro;

Laminin binding inhibitor (Lamin B-1 (442-447))

SEQ ID NO 007, Leu-Gly-Thr-Ile-Pro-Gly;

Integrin binding inhibitor (fibronectin CS-3)

SEQ ID NO 006, Gly-Arg-Gly-Glu-Ser;

Fibronectin CS-1 (1378-1382)

SEQ ID NO 005, Glu-Ile-Leu-Asp-Val;

FGF (119-126)

SEQ ID NO 012, Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu;

IGF I (30-41)

SEQ ID NO 015, Gly-Tyr-Gly-Ser-Ser-Ser-Arg-Arg-Ala-Pro-Gln-Thr;

HGF

SEQ ID NO 008, Gly-His-Lys;

Prepro-nerve growth factor (99-115)

SEQ ID NO 017, Pro-Glu-Ala-His-Trp-Thr-Lys-Leu-Gln-His-Ser-Leu-Asp-Thr-Ala-Leu-Arg;

Antagonist of platelet-derived growth factor (PDGF A-chain 194-211)

SEQ ID NO 020, Gly-Arg-Pro-Arg-Glu-Ser-Gly-Lys-Lys-Arg-Lys-Arg-Lys-Arg-Leu-Lys-Pro-Thr;

TGF alpha (34-43)

SEQ ID NO 028, Cys-His-Ser-Gly-Tyr-Val-Gly-Val-Arg-Cys;

VIP (1-12)

SEQ ID NO 002, His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg;

VEGF (GST-Exon 7 (1-20))

SEQ ID NO 019, Cys-Ser-Cys-Lys-Asn-Thr-Asp-Ser-Arg-Cys-Lys-Ala-Gly-Leu-Gly-Leu-Asn-Gly-Arg-Thr;

Endostatin (angiogenic homology region)

SEQ ID NO 029, Ser-Ala-Ala-Ser-Cys-His-His-Ala-Tyr-Ile-Val-Leu-Cys-Ile-Glu-Asn-Ser-Phe-Met-Thr-Ser-Phe-Ser-Lys;

Octreotide (Analogue of somatostatin)

Cyclic (D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr(ol).

[0056] According to the present invention, the peptide ligands can be peptide mimetics, which are analogues of the peptide ligands defined above containing one or more following modifications:

- (1) the amino acid in the peptide is replaced with a non-natural amino acid;
- (2) the amino acid in the peptide is replaced with a D form of the natural amino acid;
- (3) the C-terminal carboxylic group of the peptide is modified to an amide, a lower alkyl amide, a di-(lower alkyl) amide, a lower ester derivative, a hydroxy, or a lower alkoxy; and
- (4) the peptide is cyclized.

Amino acids

[0057] According to the present invention, the amino acid is defined as an organic compound

containing at least one carboxylic acid group and one amino group. The preferred amino acids include D or L forms of natural amino acids and non-natural amino acids.

[0058] The natural amino acids contain 20 alpha-amino acids in which an amino group and a carboxyl group are attached to a carbon. The natural amino acids, having non-polar or hydrophobic side chains, include alanine, valine, leucine, isoleucine, methionine, proline, phenylalanine, and tryptophan; having acidic side chains, include aspartic acid, and glutamic acid; having basic side chains, include lysine, arginine, and histidine; and having uncharged hydrophilic side chains, include asparagine, glutamine, glycine, serine, threonine, tyrosine, and cysteine.

[0059] The non-natural amino acids of the present invention include side chain modified amino acids, non-alpha-amino acids, and N-methyl amino acids.

[0060] Side chain modified amino acids are alpha-amino acids, wherein the side chain of each amino acid is non-natural or modified from natural amino acid. Examples of side chain modified amino acids include, but are not limited to, 2-aminobutyric acid, 1-aminocyclopropane-1-carboxylic acid, alpha-aminoisobutyric acid, biphenylalanine, p-benzoylphenylalanine, alpha-t-butylglycine, 3-cyclohexylalanine, alpha-cyclohexylglycine, (S)-2,3-diaminopropionic acid, (S)-2,3-diaminobutyric acid, 2-amino-4-phenylbutyric acid, homoserine, homotyrosine, (S)-(-)-indoline-2-carboxylic acid, beta-2-naphthyl alanine, 3-(1-naphthyl)-alanine, 3-(2-naphthyl)-alanine, octahydroindole-2-carboxylic acid, penicillamine, p-aminophenylalanine, 4-bromophenylalanine, 2-chlorophenylalanine, 3-chlorophenylalanine, 3,4-dichlorophenylalanine, 3,4-difluorophenylalanine, 2-fluorophenylalanine, 3-fluorophenylalanine, 4-fluorophenylalanine, 5-hydroxytryptophan, 4-iodophenylalanine, 4-

nitrophenylalanine, pentafluorophenylalanine, pipecolic acid, propargylglycine, thiazolidine-4-carboxylic acid, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid, 3,5-diiodotyrosine, 3-iodotyrosine, 3-nitrotyrosine, O-phosphotyrosine, diethylglycine, di-n-propylglycine, di-n-butylglycine, 1-amino-1-cyclopropane-1-carboxylic acid, 1-amino-1-cyclopentane-carboxylic acid, 1-amino-1-cyclohexane-1-carboxylic acid, and 4-hydroxyproline.

[0061] The non-alpha-amino acid is an amino acid whose amino group and carboxyl group are not attached to the same carbon. Examples of the non-alpha-amino acids include, but are not limited to, 2-aminobenzoic acid, 3-aminobenzoic acid, 4-aminobenzoic acid, 4-(aminomethyl)benzoic acid, 4-(aminomethyl)cyclohexane, beta-alanine, gamma-aminobutyric acid, 5-aminovaleric acid, 6-aminohexanoic acid, 8-aminooctanoic acid, 9-aminononanoic acid, 10-aminodecanoic acid, 11-aminoundecanoic acid, 12-aminododecanoic acid, isonipecotic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, 4-amino-5-cyclohexyl-3-hydroxypentanoic acid, and 4-amino-3-hydroxy-5-phenylpentanoic acid.

[0062] The N-alkyl amino acid is an amino acid, wherein the alpha-amino group is monoalkylated. The alkyl group includes, but is not limited to, methyl, ethyl, and propyl.

Amino alcohol

[0063] According to the present invention, the amino alcohol is a modified amino acid in which the carboxylic group is modified to a hydroxy group. The amino alcohol can be conjugated to the C-terminus of the peptide chain.

Cyclization of peptide ligand

[0064] According to the present invention, the peptide ligand can be cyclized by forming an intramolecular linkage between two amino acids or their derivatives in the peptide ligands as the above-described methods.

B. Linkage Functional Group

[0065] According to the present invention, the linkage functional group is any functional group, which can covalently link the lipid or peptide ligand to the spacer. A variety of functional groups are suitable for use in the peptide-spacer-lipid conjugates, which include, but are not limited to, those listed in Table I.

C. Spacer Group

[0066] According to the present invention, the spacer is a linear hydrophilic polymer chain containing a linkage functional group at each end of the chain for attaching the peptide and lipid. The suitable spacers in the present invention include, but are not limited to, polyglycine, polyethyleneglycol, polypropyleneglycol, polymethacrylamide, polydimethacrylamide, polyhydroxyethylacrylate, polyhydroxypropylmethacrylate, polyoxyalkene and hydrophilic peptides.

[0067] In a preferred embodiment of the present invention, the spacer is polyethylene glycol having a molecular weight between 100-10,000 daltons, more preferably between 100-5,000 daltons.

D. Lipid

[0068] According to the present invention, the lipid is either a natural or synthetic

amphipathic molecule possessing a hydrophilic and a hydrophobic portion on the same molecule, which can spontaneously form bilayer vesicles in water or can be stably incorporated into lipid bilayers.

[0069] In an embodiment of the present invention, the lipids are phospholipids including phosphodiglyceride and sphingolipid. The phosphodiglyceride has the structure with a three-carbon glycerol linked to two hydrocarbon chains at 1 and 2 position through an ester or ether linkage and a phosphoryl headgroup at 3 position. The sphingolipid consists of a hydrocarbon chain linked to the nitrogen of sphingosine through an amide bond, which is linked to a phosphoryl headgroup. The phosphoryl headgroup of the phospholipid can be phosphoryl choline, phosphoryl ethanolamine, phosphoryl serine, phosphoryl glycerol, phosphoryl inositol, and phosphatic acid. The hydrocarbon chain in phospholipid, typically, has 14-22 carbon atoms in chain length and can be saturated or at several degrees of unsaturated.

[0070] In another preferred embodiment of the present invention, the lipid is distearyl phosphatidylethanolamine (DSPE).

[0071] The lipid, which can be stably incorporated into lipid bilayers, includes but is not limited to, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerol ricinoleate, hexadecyl myristate, isopropyl myristate, amphoteric acrylic polymer, fatty acid amides, cholesterol, cholesterol ester, diacylglycerolsuccinate, diacyl glycerol, fatty acid, and the like.

[0072] In another embodiment of the present invention, the lipid is a cationic lipid, which consists of a positively charged headgroup, such as an amine, polyamine, or polylysine,

linking to a neutral lipophilic portion, such as a sterol, a hydrocarbon chain, or two hydrocarbon chains. Examples of the cationic lipids include 1, 2-dioleyloxy-3-(trimethylamino) propane (DOPAT), N-[1-(2,3-ditetradecyloxy)propyl]-N,N-dimethyl-N-hydroxyethylammonium bromide (DMRIE), N-[1-(2,3-dioleyloxy)propyl]-N,N-dimethyl-N-hydroxy ethylammonium bromide (DORIE), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 3β [N-(N',N'-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol), 3β [N-(N',N'-dimethylaminoethane)carbamyl] cholesterol (DC-Chol), and dimethylammonium bromide (DDAB).

III. Targeted Therapeutic Liposomes

[0073] The present invention further provides a targeted therapeutic liposome containing the peptide-spacer-lipid conjugates synthesized by the present method.

[0074] The targeted therapeutic liposome in the present invention comprises: (i) one or more lipids to form liposomal membranes; (ii) one or more peptide-spacer-lipid conjugates incorporated in the liposomal membranes as targeting moieties; (iii) a therapeutic or diagnostic agent incorporated in the liposome; and (iv) optionally, a hydrophilic polymer-lipid conjugate incorporated in the liposome to modify its surface.

A. Components of the Targeted Therapeutic Liposome

Lipids

[0075] The suitable lipids for the preparation of liposomes can be one or more lipids selected from a group consisting essentially of the above-defined lipids.

[0076] In an embodiment of the present invention, the lipids used in the liposomes include phospholipids and cholesterol. The preferred phospholipid is selected from a group consisting essentially of hydrogenated soybean phosphatidylcholine (HSPC), egg phosphatidylcholine (EPC), and distearyl phosphatidylcholine (DSPC).

[0077] In another embodiment of the present invention, the lipids used in the liposomes include lipid and neutral lipid, such as DOPE or cholesterol.

Hydrophilic polymer-lipid conjugates

[0078] The surface of the liposome in the present invention can be modified by hydrophilic polymer through incorporating polymer-lipid conjugates into a liposomal bilayer. The polymer-lipid conjugates in the present invention are a linear, hydrophilic polymer chain having repeating units and a linkage functional group attaching to the headgroup of a lipid. Such hydrophilic polymers include, but are not limited to, polyglycine, polyethyleneglycol, polypropyleneglycol, polymethacrylamide, polydimethacrylamide, polyhydroxyethylacrylate, polyhydroxypropylmethacrylate, polyoxyalkene, and hydrophilic peptides.

[0079] In a preferred embodiment of the present invention, the polymer in polymer-lipid conjugate is PEG having an average molecular weight between 100-10,000 Daltons, more preferably between 100-5,000 Daltons. Monomethoxy or monoethoxy PEG derivatives are also preferred polymers for the lipid conjugates.

Peptide-spacer-lipid conjugates

[0080] According to the present invention, the suitable peptide-spacer-lipid conjugates for

incorporating into liposomes are one or more peptide-spacer-lipid conjugates synthesized by the above described method.

Therapeutic agents

[0081] Various therapeutic agents, suitable for incorporating into liposomes for use in medical application, are known in the art. However, according to the present invention, the suitable therapeutic agents include, but are not limited to, natural and synthetic compounds having the therapeutic effects of being antineoplastic, anti-angiogenic, anti-bacterial, antiviral, antiparasitic, antifungal, immunoenhancing, immunosuppressive, antimigraine, antipyretic, antisera, anti-inflammatory, anticoagulant, antimetabolic, antidiuretic, antiepileptic, antimitotic, anti-arthritis, anti-arrhythmic, anti-aging, analgesic, anesthetic, hemostatic, hormonal, hormonal suppressing, hypercalcemic alleviating, hypocalcemic alleviating, hyperglycemic alleviating, hypoglycemic alleviating, muscle relaxing, neurotransmitting, psychotropic, cardiovascular, thrombolytic, and vasodilating.

[0082] According to the present invention, the suitable therapeutic agents for entrapping in the liposomes include, but are not limited to, topoisomerase I and II inhibitors, angiogenesis inhibitors, DNA-transcribing enzyme inhibitors, camptothecin and analogues, antibiotics, antiparasitics, antineoplastics, anti-inflammatory agents, antimetabolites, antimitotic agents, antitumor agents that react or bind with DNA, immune-modifying agents, oligonucleotides and polynucleotides, chemical radiation sensitizers and protectors, and photochemically active anticancer agents.

[0083] In a preferred embodiment of the present invention, the incorporated therapeutic agent

is a topoisomerase I inhibitor, including but not limited to, camptothecin and analogues that are known in the art as described by Foye W. O. (Cancer Chemotherapeutic Agents, American Chemical Society, Washington, DC, (1995)).

[0084] In another preferred embodiment of the present invention, the incorporated therapeutic agent is an anthracycline that inhibit topoisomerase II. Examples of this kind of drug are doxorubicin, daunorubicin, epirubicin, esorubicin, and idarubicin.

[0085] In another preferred embodiment of the present invention, the incorporated therapeutic agent is an antimitotic agent, such as vinblastine, navelbine, vincristine, vinglycinatate, vintrypol, and vinzolidine.

[0086] In another embodiment of the present invention, the incorporated therapeutic agent is an anti-inflammatory agent.

[0087] In another embodiment, the incorporated therapeutic agent is an angiogenesis inhibitor, such as dextrin 2-sulfate, ukrain, thalidomide, angiostatin, endostatin, and 1-[11-(dodecylamino)-10-hydroxyundecyl]-3,7-dimethyl-xanthine.

[0088] In another embodiment of the present invention, the incorporated therapeutic agents include nucleic acids, which include, but are not limited to, genes, portions of genes, oligonucleotides, RNA, and analogues thereof. The suitable genes for use as the therapeutic agents include, but are not limited to, (1) tumor suppressor genes, which may compensate for the deficient function of genes by mutation, such as *p53*, *BR1*, APC, Rb, DCC, HNPCC, NF-1, NF-2, *BRCA1*, or *BRCA2*; (2) toxin genes, which may convert inactive prodrugs into cytotoxic compounds in host cells, such as HSV-*tk*; (3) immunogenes, which may modify

cellular components of the immune system toward a tumor, or modify tumor cells to induce immune response, such as IL-2, IL-4, IL-12, or GM-CSF; (4) genes for chemosensitisation or radiosensitisation, which influence the sensitivity of the cell to chemotherapeutic agents and radiotherapy, such as liver cytochrome P450 gene, *CYP2B1*, or *tk* gene; (5) genes or the protein encoded genes, which modulate the apoptotic process of cells, such as TNF/TNFR1, Apo3L/DR3, Apo2L/DR4 or 5, cytochrome c, *TP53*, *E1A*, bax, bcl-x_s, apoptin, *bcl-2*, surviving, XIAP, IAP-1, or IAP-2; and (6) genes corresponding to drug resistance, such as MDR1.

[0089] The oligonucleotides include, but are not limited to, antisense oligonucleotides, which may bind to mRNA or DNA to regulate translation or transcription of the genes. The target genes of the antisense oligonucleotides include, but are not limited to, mutants of tumor suppressor genes (e.g., *p53*, *BR1*, *E1A*, and *BRCA1*); oncogenes (e.g., *k-ras*, *c-myc*, and *c-fos*); genes of growth factors (e.g., IGF 1, PDGF, acidic and basic FGF, and TGF β); genes encoding the proteins that respond to multiple drug resistance (e.g., MDR1).

Diagnostic agents

[0090] The diagnostic agents used in the present invention include, but are not limited to, gamma emitting radionucleotides for imaging agents in gamma scintigraphy, radiopaque materials for computed tomography, and paramagnetic metal ions for magnetic resonance.

[0091] In a preferred embodiment of the present invention, the gamma-emitting radionuclides are ⁶⁷Ga, ¹¹¹In, and ^{99m}Tc.

[0092] In a preferred embodiment of the present invention, the paramagnetic metal ion is Gd.

B. Liposomes

[0093] According to the present invention, the liposomes suitable for preparing targeted therapeutic liposomes are spherical particles, which consist of bilayer membranes formed by one or more lipids and one or more aqueous compartments are enclosed therein.

[0094] According to the present invention, the liposome size ranges from approximately 30 nm to approximately 1000 nm, depending on the target organs or tissues and the therapeutic agents to be delivered. For example, the preferred liposome size, for a blood stream delivery therapeutic agent, is in a range of approximately 50 nm to approximately 150 nm; and for a directly applied therapeutic agent to tissue or tumor site, the preferred liposome size is in a range of approximately 30 nm to approximately 80 nm.

[0095] In a preferred embodiment of the present invention, the liposomes are composed of (1) natural phospholipids that are extracted from soybean or egg, (2) cholesterol, (3) polymer-lipid conjugate.

[0096] In another embodiment of the present invention, the liposomes are cationic liposomes including the components of a cationic lipid and one or more neutral lipids, such as DOPE and cholesterol.

C. Preparation of Targeting Therapeutic Liposomes

[0097] According to the present invention, the targeted therapeutic liposomes can be prepared by incorporating peptide-spacer-lipid conjugates synthesized by the present method into a

therapeutic agent containing liposomes.

[0098] The liposomes of the present invention can be prepared by a variety methods as known in the art, for example, Gregoriadis, G., ed. *Liposome Technology*, Vols., I, II, III, CRC Press, Boca Raton, Fla., 1984; Szoka, F., et al., *Ann. Rev. Biophys. Bioeng.* 9:476 (1980); and New, R.R.C., *Liposomes, a Pratical Approach*, Oxford IRL Press, New York, 1990, which are incorporated herein by reference.

[0099] In an embodiment of the present invention, the liposomes are prepared by hydrating a lipid film to produce initial multilamellar vesicles, which are subject to extrusion or homogenization method to reduce the size of the vesicles. Typically, a lipid (or lipid combination) with or without hydrophilic polymer-lipid conjugate in an organic solvent is evaporated and dried in vacuum to form a thin film in a vessel. The lipid film is hydrated in an aqueous solution by vortexing to form the initial multilamellar vesicles. The aqueous vesicles are then preformed by several cycles of freezing and thawing. The suspended multilamellar vesicles are downsized by either a membrane extrusion or an homogenization method, as described in Hope *et al*, *Biochim. Biophys. Acta.*, 812, 55-65 (1985); Mayhew *et al*, *Biochim. Biophys. Acta.*, 775, 169-174 (1984); and Brandl *et al.*, in Gregoriadis, G., ed. *Liposome Technology*, 2nd ed., Vol. I, CRC Press, Boca Raton, Fla., 1992, pp. 49-65, which are incorporated herein by reference.

[0100] Loading of a therapeutic or a diagnostic agent to the liposomes includes the methods of loading water-soluble, hydrophobic, and ionic compounds. Water-soluble compounds generally are encapsulated in liposomes by dissolving the agent in an aqueous solution and mixing with a lipid film. Hydrophobic agents can be entrapped into the liposomes or

incorporated into the lipid bilayer by dissolving the agent with a lipid or lipid combination in a suitable organic solvent and then evaporating the solvent to produce a thin film. Methods for loading ionic agents can be performed by pH, ionic gradient methods as described in Mayer *et al.*, *Biochemistry*, 27, 2053-2060 (1988) and Haran, G. *et al.*, *Biochim. Biophys. Acta.*, 1151, 201-215 (1993), which are incorporated herein by reference.

[0101] DNA can be incorporated to liposomes by several ways, which include, but not limited to, (1) entrapping the DNA into liposomes; (2) forming a lipoplex (a DNA-liposome complex); and (3) forming a lipopolyplex (a complex of liposome, polycationic polymer and DNA). The methods for preparing these DNA incorporated liposomes are known in the art, for example, Hug P and Sleight R.G., *Biochim. Biophys. Acta.*, 1097:1-17 (1991); Nabel, G.L. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 90, 11307-11311 (1993); Gao, X. and Huang L., *Biochemistry*, 35, 1027-1036 (1996); and Whitmore *et al.*, *Gene Ther.*, 6, 1867-1875 (1999), which are incorporated herein by reference.

[0102] According to the present invention, incorporating peptide-spacer-lipid conjugates into liposomal membranes can be achieved by incubating micelles of peptide-spacer-lipid conjugate / methoxypolyethylene glycol-distearyl phosphatidylethanolamine (mPEG-DSPE) with a therapeutic agent-containing liposome at a temperature higher than the transition temperature of the lipid membrane. In general, the dried lipid film of peptide-spacer-lipid conjugate / mPEG-DSPE is hydrated in an aqueous buffer, at a concentration higher than the critical micellar concentration of the conjugates, with gentle swirling of the mixture at a raised temperature. After the lipid film is melted and the mixture becomes a clear micellar solution, the micellar solution is then transferred into the therapeutic agent-containing

liposomes at a temperature higher than the transition temperature of the liposomal membrane for a period of time to complete the insertion. The solution is then passed through a size exclusion column to separate micelles and targeted liposomes. Fractions of micelles and targeted liposomes are pooled separately for quantitative analyses.

[0103] According to the present invention, the transition temperature of lipid membrane effects the preparation of targeted therapeutic liposomes. The suitable transition temperature of liposomes in the present invention is in a range of approximately 3°C - approximately 70°C.

[0104] The following examples are used for illustration, but not for limiting the present invention.

Example 1: Preparation of Amino Alcohol

[0105] *Preparation of Fmoc-Thr(tBu)-alcohol (Fmoc-Thr(tBu)-ol).* Fmoc-Thr(tBu)-OH (1 eq, 0.663 g, 1.67 mmol) was suspended in 2 ml of ethylene glycol dimethyl ether (DME) and chilled below -15°C under nitrogen. After addition of N-methylmorpholine (1 eq, 0.19 ml, 1.67 mmol) and isobutyl chloroformate (1 eq, 0.22 ml, 1.67 mmol), the mixture was stirred at -15°C. After 5 min of stirring, the precipitate was removed, and a suspension of NaBH₄ (3 eq) in 5 ml of water was added and stirred for another 1 hr. At the end of the reaction, 40 ml of water was added. The mixture was extracted with DCM (20 ml x 3), and the combined organic layers were washed with 5 % NaHCO₃, followed by rinsing with brine (e.g., NaCl), and being dried over anhydrous Na₂SO₄ (or MgSO₄). The solvent was evaporated. Crude Fmoc-Thr (tBu)-alcohol was purified by silica gel column chromatography using DCM as an

eluent: ¹H-NMR (CDCl₃) δ (ppm): 1.16 (3H, d, J = 6.2 Hz, CHCH₃), 1.20 (9H, s, tBu), 2.88 (1H, broad, OH), 3.61 (1H, broad, CHCH₂OH), 3.66 (2H, broad, CHCH₂OH), 3.94 (1H, m, CHCH₃), 4.22 (1H, t, J = 6.8 Hz, CHCH₂CO), 4.40 (2H, m, CHCH₂CO), 5.28 (1H, d, J = 7.5 Hz, NH), 7.30 (2H, d, J = 7.4 Hz, aromatics), 7.38 (2H, t, J = 7.2 Hz, aromatics), 7.59 (2H, d, J = 7.4 Hz, aromatics), 7.74 (2H, d, J = 7.4 Hz, aromatics).

Example 2: Preparation of Peptidyl-Resins

[0106] The peptidyl-resins were prepared according to the Merrifield solid phase synthesis techniques (See Steward and Young, *Solid Phase Peptide Synthesis*, 2nd edition, Pierce Chemical Company, Rockford, (1984) and Merrifield, *J. Am. Chem. Soc.* 85, 2149-2154 (1963)). In the present invention, a Wang resin, a 2-chlorotrityl chloride resin, and a Rank amide resin in the Fmoc synthetic techniques were used. The Wang resin was used to synthesize the peptidyl moieties in which they have carboxylic acid moiety at a C-terminus. A 2-Chlorotrityl chloride resin was used to synthesize the peptidyl moieties in which they have Pro, Cys, or amino alcohols at a C-terminus. A Rink amide resin was used to synthesize the peptidyl moieties in which they have amide at a C-terminus. Applications of these resins in SPPS were described in the art, for examples, S.-S. Wang, *J. Am. Chem. Soc.*, 95, 1328 (1973) and G. Lu, *et al.*, *J. Org. Chem.*, 46, 3433 (1981) for the Wang resin; K. Barlos, *et al.*, *Int. J. Peptide Protein Res.*, 37, 513 (1991) and K. Barlos, *et al.*, *Int. J. Peptide Protein Res.*, 38, 562 (1991) for 2-Chlorotrityl chloride resin; H. Rink, *Tetrahedron Lett.*, 28, 3787 (1987); M. S. Bematowicz, *et al.*, *Tetrahedron Lett.*, 30, 4645 (1989) for the Rink resin.

[0107] The amino groups for peptide chain formation were protected by an Fmoc group. T-butyl was used as the side chain protecting groups for tyrosine, serine, threonine, glutamic

acid, and aspartic acid; Trt was used for asparagine and histidine; Boc was used for lysine and tryptophan; Pbf was used for arginine; and Acn was used for cysteine.

[0108] In general, the peptides were assembled according to the cycle consisting of (1) 30 min of removing Fmoc protecting group with 20% piperidine-DMF and (2) 2 hr of coupling of the Fmoc amino acid derivative (2eq) with DIPCDI (2eq) and HOBt (2eq) in DMF. The coupling reaction was repeated when the resin became positive to the Kaiser test (Kaiser *et al.*, 1970). After the desired peptide was assembled, a small portion of peptidyl-resin was cleaved by a cleavage cocktail of TFA, chloroform, thioanisole, EDT, and anisole. The cleaved peptide was purified by HPLC and identified by MS. The constructed peptides are listed as below:

EGF

SEQ ID NO 009, H-Cys(Acn)-Met-His-Ile-[Gly]-Glu-Ser-Leu-Asp-Ser-Tyr-Thr-Cys(Acn)-OH

MS expected: 1543.7, MS found: 1543.8;

FGF I

SEQ ID NO 011, H-Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys-Lys-Pro-OH

MS expected: 1289.7, MS found: 1290.4;

Laminin binding inhibitor

SEQ ID NO 007, H-Leu-Gly-Thr-Ile-Pro-Gly-OH

MS expected: 556.3, MS found: 557;

Integrin binding inhibitor

SEQ ID NO 006, H-Gly-Arg-Gly-Glu-Ser-OH

MS expected: 504.2, MS found: 505;

Fibronectin CS-1 fragment

SEQ ID NO 005, H-Glu-Ile-Leu-Asp-Val-OH

MS expected: 587.6, MS found: 587.6;

FGF (119-126)

SEQ ID NO 012, H-Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu-OH

MS expected: 993.2, MS found: 993.3;

IGF I (30-41)

SEQ ID NO 014, H-Gly-Tyr-Gly-Ser-Ser-Ser-Arg-Arg-[Arg-]Ala-Pro-Gln-Thr-OH

MS expected: 1266.4, MS found: 1267;

HGF

SEQ ID NO 008, H-Gly-His-Lys-OH

MS expected: 340.3, MS found: 340.1;

Prepro nerve growth factor (99-115)

SEQ ID NO 017, H-Pro-Glu-Ala-His-Trp-Thr-Lys-Leu-Gln-His-Ser-Leu-Asp-Thr-Ala-Leu-Arg-OH

MS expected: 2003.2, MS found: 2003.0;

Analogue of somatostatin (Octreotide)

cyclic H-(D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr(ol)

MS expected: 1019.2, MS found: 1019.4; and

Analogue of somatostatin

cyclic H-(D)Phe-Cys-Phe-Gly-Lys-Thr-Cys-Thr(ol)

MS expected: 890, MS found: 890.

Example 3: Synthesis of End-Group Functionalized PEG Derivatives

Carboxyl-PEG and its active esters

[0109] Carboxyl-PEG. PEG₂₀₀₀ (8.6 g) and potassium *tert*-butoxide (20 g) were dissolved in 300 ml *tert*-butyl alcohol and warmed to 40°C. Ethyl bromoacetate (10 ml) was added over a period of 20 min. The mixture was stirred for 2 hr and then evaporated to remove solvent. The residue was hydrolyzed in 200 ml of 1 N NaOH and stirred at room temperature for 2 hrs. At the end of hydrolysis, the pH of the mixture was adjusted to 2 and extracted by CHCl₃ (2 x 200 ml). The combined extract was washed with water, dried over anhydrous MgSO₄,

evaporated to concentrate and dried in a vacuum. A white Carboxyl-PEG powder was obtained and yielded 6.88 g. $^1\text{H-NMR}$ (CDCl_3) δ (ppm): 3.66 (s, $\text{O-CH}_2\text{CH}_2\text{-O}$), 4.13 (s, $\text{HO-C(O)-CH}_2\text{-O}$).

[0110] *PEG-oxybenzotriazole*. HOBt (2.6 mmol), DIPCDI (1.91 mmol), and carboxyl- PEG_{3000} (0.87 mmol) were mixed in 4 ml DMF and stirred at room temperature under nitrogen for 20 min. The mixture was applied to conjugate with peptidyl-resin without further purification of the PEG-oxybenzotriazole.

[0111] *Activation of carboxyl-PEG with dicyclohexylcarbodiimide (DCC)*. DMAP (1.91 mmol), DCC (1.91 mmol) and carboxyl- PEG_{3000} (0.87 mmol) were mixed in 4 ml DMF and stirred at room temperature under nitrogen for 20 min. The mixture was applied to conjugate with peptidyl-resin without further purification of the activated carboxyl-PEG.

[0112] *Succinimidyl ester of carboxyl-PEG (Su-OC(O)-PEG)*. HNS (2.6 mmol) and EDC (2.6 mmol) were added into a mixture of carboxyl- PEG_{2000} (0.87 mmol) in 4 ml DMF, and stirred at room temperature under nitrogen overnight. At the end of the reaction, the mixture was evaporated to remove the solvent. The residue was added to 10 ml H_2O and extracted with 20 ml DCM for three times. The combined extract was washed with saturated brine (e.g., NaCl), dried over anhydrous MgSO_4 , and then concentrated and dried in vacuum. The crude product was precipitated by ether and further purified from isopropanol/ether to yield 45%. $^1\text{H-NMR}$ (CDCl_3) δ (ppm): 2.87 (s, $\text{O-N(C(O)CH}_2)_2$), 3.66 (s, $\text{O-CH}_2\text{CH}_2\text{-O}$), 4.53 (s, $\text{-CH}_2\text{O-C(O)-OSu}$).

[0113] *p-Nitrophenyl carbonate of PEG (pNP-O-C(O)-PEG)*. P-nitrophenyl chloroformate

(2.22 g) was added into a mixture of PEG₂₀₀₀ (10 g) and TEA (1.31 ml) in 40 ml DCM, and stirred at room temperature under nitrogen overnight. At the end of stirring, the mixture was filtered to remove TEA-HCl salt and evaporated to remove solvent. The crude product was precipitated by isopropyl ether and crystallized from ethyl acetate and ethyl ether twice. ¹H-NMR (CDCl₃) δ (ppm): 3.66 (s, O-CH₂CH₂-O), 3.80 (4H, s, O-CH₂CH₂-OC(O)OC₆H₄NO₂), 4.44 (4H, s, O-CH₂CH₂-OC(O)OC₆H₄NO₂), 7.38 & 8.28 (8H, dd, -OC(O)OC₆H₄NO₂).

[0114] *Tos-PEG* (CH₃C₆H₄S(O₂)O-PEG). P-toluenesulfonyl chloride (2.29 g) was added into a mixture of PEG₂₀₀₀ (10 g) and pyridine (1.21 ml) in 15 ml DCM and stirred at room temperature under nitrogen overnight. At the end of the reaction, the mixture was evaporated to remove solvent. A white crude product was precipitated by a mixed solvent of isopropanol/ isopropyl ether at 1/1.3 ratio in ice bath. The crude product was crystallized twice by ethyl acetate /ethyl ether at 1/1 volume ratio. ¹H-NMR (CDCl₃) δ (ppm): 2.34 (6H, s, -OSO₂C₆H₄CH₃), 3.66 (s, O-CH₂CH₂-O), 4.15 (4H, s, O-CH₂CH₂-OSO₂C₆H₄CH₃), 7.16 & 7.79 (8H, dd, -OSO₂C₆H₄CH₃).

Example 4: Preparation of the Spacer-Lipid Conjugate

[0115] *Preparation of HOC(O)-PEG₂₀₀₀-C(O)NH-DSPE*. Su-OC(O)-PEG₂₀₀₀ (0.6 mmol) was added to a mixture containing DSPE (0.449g , 0.6 mmol), TEA (0.2 ml, 1.4 mmol) and DMF (5 ml). The mixture was stirred in a temperature range of 40°C to 45°C for a duration of 4 hrs. The product was confirmed by thin layer chromatography using a solution of chloroform/ methanol/water (3: 1: 0.1 v/v).

[0116] *Preparation of Pnp-O -PEG₂₀₀₀-C(O)NH-DSPE*. pNP-O-C(O)-PEG₂₀₀₀ (0.6 mmol)

was added to a mixture containing DSPE (0.45 g, 0.6 mmol), TEA (0.6 mmol) and chloroform (10 ml). The mixture was stirred in a temperature range of 40°C to 45°C for a duration of approximately 2 hrs. The product was confirmed by thin layer chromatography using a solution of chloroform/ methanol/water (3: 1: 0.1 v/v).

Example 5: Conjugation of the Spacer to the Peptidyl Resin

[0117] *Preparation of HOC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin.* H-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin (0.13 mmol) obtained from solid phase peptide synthesis by 2-chlorotrityl chloride resin was treated with 5 ml of 20% piperidine in DMF to remove the Fmoc protection group from the N-terminus of peptidyl-resin. After removing Fmoc, the resin was washed with DMF (5 ml x 3). A mixture of PEG-oxybenzotriazole, obtained by mixing HOBt (0.8 mmol), DIPCDI (0.8 mmol) and carboxyl-PEG₆₀₀ (0.4 mmol) in 5 ml DMF, was added to H-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin. The coupling reaction proceeded for 2 hrs at room temperature with shaking. The completion of the reaction was checked by Kaiser test. At the end of the reaction, the excess reagents and byproducts were washed away. A small portion of the HOC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin was cleaved and identified by NMR and MS spectroscopy. The ¹H NMR (CD₃OD) showed ethylene glycol peak at δ 3.5 ppm and the proton of the peptide. In mass spectrum, a bell shape of molecular distribution was observed due to the different molecular mass of PEG. The measured central molecular weight of 1763.5 for HOC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp-Lys-Thr-Cys(Acm)-

Thr-ol virtually matches the calculated molecular weight of 1763.6.

[0118] *Preparation of HOC(O)-PEG₂₀₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin.* The reaction conditions and procedures were performed as described above, except that carboxyl-PEG₂₀₀₀ was used to replace carboxyl-PEG₆₀₀.

[0119] *Preparation of HOC(O)-PEG₃₀₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin.* The reaction conditions and procedures were performed as described above, except that carboxyl-PEG₃₀₀₀ was used to replace carboxyl-PEG₆₀₀.

[0120] *Preparation of pNP-OC(O)-PEG₂₀₀₀-OC(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin.* H-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin (0.13 mmol) obtained from solid phase peptide synthesis was treated with 5 ml 20% piperidine in DMF to remove Fmoc protection group from N-terminus of peptidyl-resin. After removing Fmoc, the resin was washed with DMF (5 ml x 3). A mixture of pNP-O-C(O)-PEG₂₀₀₀ (0.39 mmol) and TEA (1.15 mmol) in 5 ml DMF was added to H-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin. The coupling reaction proceeded at room temperature overnight. The completion of the reaction was verified using a Kaiser test. At the end of the reaction, the excess reagents and byproducts were washed away. A small portion of the assembled HO-PEG₂₀₀₀-OC(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp-Lys-Thr-Cys(Acm)-Thr-ol conjugate was cleaved from the resin and identified by ¹H NMR spectroscopy. The ¹H NMR (CD₃OD) showed ethylene glycol peak at δ 3.5 ppm and the proton of the peptide.

Example 6: Conjugation of Lipid to the Spacer-Peptidyl Resin

[0121] Preparation of DSPE-NHC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin. *HOC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin (0.13 mmol) in 2 ml DMF was added with NHS (0.4 mmol) and EDC (0.4 mmol) and then the mixture is shaken at room temperature for 4 hrs. Subsequently, DSPE (0.26 mmol) was added to couple with the HOC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin (0.13 mmol) in a mixed solvent (4 ml chloroform and 0.5 ml TEA) at 55°C for overnight. After the reaction, the resin was washed with chloroform, DMF, and MeOH and subjected to cleavage.*

Example 7: Conjugation of Spacer-Lipid to the Peptidyl-Resin

[0122] Preparation of DSPE-NHC(O)-PEG₂₀₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin. *HOC(O)-PEG₂₀₀₀-DSPE (0.4 mmol) in DMF (5 ml) was added DIPC DI (0.8 mmol) and HOBT (0.8 mmol). The solution was stirred at room temperature for 30 min and then added to peptidyl resin. The coupling reaction proceeds for approximately 2 hrs at approximately room temperature with shaking. The completion of the reaction was verified using a Kaiser test. At the end of the reaction, the excess reagents were wash off and the resin was subject to cleavage.*

Example 8: Cleavage of the Lipid-Spacer-Peptidyl Resin

[0123] The DSPE-NHC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-ol-resin was cleaved by a cleavage cocktail (50% TFA, 45%

CHCl₃, 3.75% anisole, and 1.25% EDT). The cleaved mixture was shaken at room temperature for an additional 10 min to completely remove the protection groups. The mixture was cooled in an ice bath, and then cold ether was added to precipitate the product. The precipitate was spun and washed with cold ether three times. The crude product was purified by liquid chromatography with C8 silica column, and eluted with a methanol gradient (0 to 85% v/v) in water to yield a white solid powder (200 mg /g of resin). The DSPE-NHC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp-Lys-Thr-Cys(Acm)-Thr-ol was identified by ¹H NMR and MS. ¹H-NMR (MeOH) δ (ppm): 0.90 (6H, t, CH₃-(CH₂)_n-), 1.29 (56H, br. s, -(CH₂)_n-), 1.97 (6H, s, -NHC(O)-CH₃), 1.59 (4H, m, CH₂-CH₂CH₂-C(O)O-), 2.33 (4H, t, -CH₂CH₂-C(O)O-), 3.63 (268H, s, -O-(CH₂CH₂)_n-O-), 5.23 (1H, s, *sn*2 proton of glycerol), 6.85-8.50 (15H, aromatic protons of phenyl, indol). The measured central molecular weight of 2451 of the mass spectrum matches the calculated molecular weight of 2450.

Example 9: Disulfide Cyclization of Lipid-Spacer-peptide conjugate

[0124] DSPE-NHC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys(Acm)-Phe-(D)Trp-Lys-Thr-Cys(Acm)-Thr-ol was dissolved in methanol at a concentration less than 1.0 mg/ml with 10 equivalent of I₂ (40 μl of 20 % I₂ in 1 ml of methanol) and the mixture is shaken at room temperature for 1 hour. The solution was transferred into a dialysis tube, such as a Spectra/PorTM dialysis tube (MWCO 2,000), and dialyzed against water at 4°C (3 x 1000 ml, 8-16h per period). The solution was then lyophilized to give a white fluffy solid. The measured central molecular weight 2351 of the mass spectrum of cyclized DSPE-NHC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr-ol matches the calculated molecular weight of 2351. ¹H-NMR

(MeOH) δ (ppm): 0.90 (6H, t, CH₃-(CH₂)_n-), 1.29 (56H, br. s, -(CH₂)_n-), 1.59 (4H, m, CH₂-CH₂CH₂-C(O)O-), 2.31 (4H, t, -CH₂CH₂-C(O)O-), 3.64 (268H, s, -O-(CH₂CH₂)_n-O-), 5.22 (1H, s, *sn*2 proton of glycerol), 6.85-8.50 (15H, aromatic protons of phenyl, indol).

Example 10: Preparation of Therapeutic Agent Contained Liposomes

[0125] Lipid components of HSPC (0.121 mmol)/Chol/mPEG-DSPE (10:7:0.4 molar ratio) were dissolved in chloroform/methanol (1:1) and evaporated to remove any organic solvent, and then dried in a vacuum to obtain a lipid film. The lipid film was subjected to a vigorous vortex for hydration in a buffer of 150 mM ammonium sulfate, and a freeze-thaw cycle was proceeded for 10 times. The mixture was extruded through double-stacked polycarbonate membranes (pore sizes from 400 to 100 nm) using an extruding device from LiposofastTM (Ottawa, Canada) to produce liposomes. The outside buffer solution of liposomes was changed to 300 mM histidine by passing through a gel filtration column. Loading of DOX was performed by mixing liposomes with a solution of 10 mg DOX in 1 ml 300 mM histidine at 65°C for 1 hr. Free DOX was then removed by passing through a gel filtration column in the buffer of HEPES 25 mM and NaCl 150 mM (pH 7.2). The phospholipid concentration of the liposomes was determined by their phosphorus content, the vesicle size was determined by dynamic laser scattering, and DOX content was determined by UV. The liposomes had a mean vesicle size of 135 nm with a standard deviation of < 25% and a normal size distribution. The DOX content in the liposomes reaches 0.28 drug/lipid molar ratio.

Example 11: Preparation of Targeted Therapeutic Liposomes

[0126] DSPE-NHC(O)-PEG₆₀₀-C(O)NH-(D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr-ol/mPEG-DSPE (0.004/0.012 mmol) was dissolved in 3 ml chloroform/methanol (1:1) and evaporated to make a dried lipid film. The dried lipid film was hydrated in 1 ml solution of HEPES 25 mM and NaCl 150 mM (pH 7.2) with gentle swirling of the mixture at 60°C. After the lipid film melted and the mixture turned to a clear micellar solution, the micellar solution was then transferred into 4 ml DOX contained liposomes (contained 0.21 mmol total lipids and 0.058 mmol DOX·HCl) at 60°C for 4 hours to complete the insertion. The solution is then passed through a gel filtration column, such as SepharoseTM CL-4B (Pharmacia BiotechTM) column, to separate micelles and targeted therapeutic liposomes. Fractions of micelles and targeted therapeutic liposomes were pooled separately for quantitative analyses. The inserted peptide-PEG-lipid conjugates in liposomes were about 1% of the total lipid of liposomes.

[0127] Information as herein shown and described in detail is fully capable of attaining the above-described object of the invention, the presently preferred embodiment of the invention, and is, thus, representative of the subject matter, which is broadly contemplated by the present invention. The scope of the present invention fully encompasses other embodiments which may become obvious to those skilled in the art, and is to be limited, accordingly, by nothing other than the appended claims, wherein reference to an element in the singular is not intended to mean "one and only one" unless explicitly so stated, but rather "one or more." All structural, compositional, and functional equivalents to the elements of the above-described preferred embodiment and additional embodiments that are known to those of

ordinary skill in the art are hereby expressly incorporated by reference and are intended to be encompassed by the present claims.

[0128] Moreover, no requirement exists for a device or method to address each and every problem sought for resolution by the present invention, for such to be encompassed by the present claims. Furthermore, no element, component, or method step in the present disclosure is intended to be dedicated to the public regardless of whether the element, component, or method step is explicitly recited in the claims. However, it should be readily apparent to those of ordinary skill in the art that various changes and modifications in form, reagents, and synthesis detail may be made without departing from the spirit and scope of the inventions as set forth in the appended claims. No claim herein is to be construed under the provisions of 35 U.S.C. §112, sixth paragraph, unless the element is expressly recited using the phrase “means for.”

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